

Att. Docket No. REG 670A-US  
USSN 09/868,677 filed October 1, 2001  
Amendment And Response To December 7, 2001  
Notification Of Missing Requirements

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which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B (SEQ ID NO: 11), followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B [SEQ ID NO: 11]), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D [SEQ ID NO: 11]), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D [SEQ ID NO: 11]), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E [SEQ ID NO: 11]). This molecule was subcloned into the HindIII and NotI polylinker sites in the expression vector pRG763 and was designated pRG763-m(Ephrin-B2)2-Fc. As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

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#### REMARKS


This amendment is being made merely to insert the sequence identifiers. Applicants submit herewith as Exhibit 1: a copy of the December 7, 2001 Notification; Exhibit 2: copy of Transmittal of Sequence Listing submitted concurrently to the U.S. Patent and Trademark Office, Box Sequence, P.O. Box 2327, Arlington, VA 22202; Exhibit 3: Marked-Up Versions of amended pages 4, 7, 26, 27, 29, 31, 32, 37, 38, 39, 41, 42, and 44.

Applicants have directed the subject Sequence Listings submitted with the Transmittal of Sequence Listing be added to the specification.

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No fee is deemed necessary in connection with the filing of this Response.  
However, if any fee is required, authorization is hereby given to charge the amount  
of any such fee to Deposit Account No. 18-0650.

Respectfully submitted,



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cells. The invention also provides for nucleic acids encoding such polypeptide ligands, and both prokaryotic and eukaryotic expression systems for producing such polypeptide ligands. According to the invention, soluble forms of the polypeptide ligands described herein may be used to promote biological responses in receptor-expressing cells. In particular, a general method is described herein which produces fusion polypeptide ligands that may then be clustered, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

#### DESCRIPTION OF THE FIGURES

<sup>(SEQ ID NOS: 1 and 2)</sup> <sup>(SEQ ID NO: 1)</sup> <sup>(SEQ ID NO: 2)</sup>  
Figure 1A-1E<sup>1</sup> Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-FD-Fc.

<sup>(SEQ ID NOS: 3 and 4)</sup> <sup>(SEQ ID NO: 3)</sup> <sup>(SEQ ID NO: 4)</sup>  
Figure 2A-2E<sup>1</sup> Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-FD-Fc.

<sup>(SEQ ID NOS: 5 and 6)</sup> <sup>(SEQ ID NO: 5)</sup> <sup>(SEQ ID NO: 6)</sup>  
Figure 3A-3E<sup>1</sup> Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-Fc-FD.

<sup>(SEQ ID NOS: 7 and 8)</sup> <sup>(SEQ ID NO: 7)</sup> <sup>(SEQ ID NO: 8)</sup>  
Figure 4A-4E<sup>1</sup> Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-Fc-FD.

Figure 5 - Molecular Weight Analysis of Ang-1-FD-Fc-FD protein. SDS

PAGE analyses showing a band running at about 210kD under non-reducing conditions (lane 3) and a band running at about 85kD under reducing conditions (lane 7).

blocking Ang1\*-mediated phosphorylation of Tie-2, as shown in Figure 11.

(SEQ ID NO: 9 and 10) (SEQ ID NO: 9) (SEQ ID NO: 10)  
Figure 14A-14E<sup>1</sup> Nucleic acid sequence<sup>1</sup> and deduced amino acid sequence<sup>1</sup>  
of Ephrin-B1-Ephrin-B1-Fc.

5 (SEQ ID NO: 11 and 12) (SEQ ID NO: 11) (SEQ ID NO: 12)  
Figure 15A-15E<sup>1</sup> Nucleic acid sequence<sup>1</sup> and deduced amino acid sequence<sup>1</sup>  
of Ephrin-B2-Ephrin-B2-Fc.

10 Figure 16 - Comparison of Ephrin-B1-Fc, Ephrin-B1-Ephrin-B1-Fc, Ephrin-B2-Fc and Ephrin-B2-Ephrin-B2-Fc in standard EphB2 phosphorylation assays. COS cells were serum-starved and then left untreated (UT), lane 1, or were treated with unclustered and clustered Ephrin-B1-Fc (Efn-B1), lanes 2 and 3. COS cells were also treated with unclustered and clustered Ephrin-B1-Ephrin-B1-Fc (Efn-B1 DD), lanes 4 and 5. In addition cells were likewise  
15 treated with unclustered and clustered Ephrin-B2-Fc (Efn-B2), lanes 6 and 7 and with unclustered and clustered Ephrin-B2-Ephrin-B2-Fc (Efn-B2 DD), lanes 8 and 9. The extent of EphB2 phosphorylation was assessed by anti-phosphotyrosine western blotting (upper panels) and the relative amounts of EphB2 in each lane was determined by anti-EphB2 western blotting  
20 (lower panels).

Figure 17 - Ang1\*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were stimulated with 0.4 µg/ml Ang1\* or 0.2 µg/ml or 0.4 µg/ml stable  
25 CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1\* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells.

30 Figure 18 - Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were treated with 0.2

Institute, Inc.) with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5' end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

**Example 1: Construction of the Ang-1-FD-FD-Fc, Ang-2-FD-FD-Fc, Ang-1-FD-Fc-FD, and Ang-2-FD-Fc-FD nucleic acid molecules.**

**Ang-1-FD-FD-Fc:** Ang-1-FD-FD-Fc consists of a trypsin signal sequence at its amino terminus to allow for secretion (bases 1-45 of Figure 1A) followed by the angiopoietin-1 fibrinogen domain (FD) (bases 46-690 of Figure 1A-Figure 1B), a short bridging sequence consisting of the amino acids Gly-Pro Ala-Pro (bases 691-702 of Figure 1B), a second angiopoietin-1 FD (bases 703-1750 of Figure 1B-Figure 1D), another bridging sequence consisting of the amino acids Gly-Pro-Gly (bases 1351-1359 of Figure 1D), and the coding sequence for the Fc portion of human IgG1 (bases 1360-2058 of Figure 1D-Figure 1E).

**Ang-2-FD-FD-Fc:** The Ang-2-FD-FD-Fc nucleic acid molecule was similarly constructed. It consists of a trypsin signal sequence (bases 1-45 of Figure 2A), an angiopoietin-2 FD (bases 46-690 of Figure 2A- Figure 2B), a bridging amino acid sequence Gly-Gly-Pro-Ala-Pro (bases 691-705 of Figure 2B), a second angiopoietin-2 FD (bases 706-1353 of Figure 2B-Figure 2D), another bridging amino acid sequence Gly-Pro-Gly (bases 1354-1362 of Figure 2D), and the coding sequence for the Fc portion of human IgG1 (bases 1363-2061 of Figure 2D-Figure 2E).

**Ang-1-FD-Fc-FD:** The Ang-1-FD-Fc-FD consists of a trypsin signal sequence (bases 1-45 of Figure 3A), an angiopoietin-1 FD (bases 46-690 of Figure 3A-3B), the bridging amino acid sequence Gly-Pro-Gly (bases 691-699 of Figure

[SEQ ID NO: 5]  
 3B), the coding sequence for the Fc portion of human IgG1 (bases 700-1395 of Figure 3B-3D), [SEQ ID NO: 5] another bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1396-1419 of Figure 3D), [SEQ ID NO: 5] and a second angiopoietin-1 FD (bases 1420-2067 of Figure 3D-Figure 3E). [SEQ ID NO: 5]

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**Ang-2-FD-Fc-FD:** The Ang-2-FD-Fc-FD nucleic acid molecule consists of a trypsin signal sequence (bases 1-45 of Figure 4A), [SEQ ID NO: 7] an angiopoietin-2 FD domain (bases 46-690 of Figure 4A-Figure 4B), [SEQ ID NO: 7] the bridging amino acid sequence Gly-Gly-Pro-Gly (bases 691-702 of Figure 4B), [SEQ ID NO: 16] [SEQ ID NO: 7] the coding sequence [SEQ ID NO: 7] for the Fc portion of human IgG1 (bases 703-1398 of Figure 4B- Figure 4D), [SEQ ID NO: 15] the bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1399-1422 of Figure 4D), [SEQ ID NO: 7] and a second angiopoietin-2 FD (bases 1423-2067 of Figure 4D-Figure 4E). [SEQ ID NO: 7]

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15 **Example 2: Characterization of Ang-1 FD-Fc-FD protein.**

**Molecular Weight Analysis:** The predicted molecular weight for Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses of COS cell-derived protein described *infra* confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions (Figure 5). Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To

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it is derived, and the mutant version of angiopoietin-1 called Ang1\* that consists of the N-terminal of angiopoietin-2 fused to the coiled-coil domain and fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. (See PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc., especially Figure 27, which is hereby incorporated by reference).

Both angiopoietin-1 and Ang1\* require extensive, expensive and labor-intensive purification schemes that result in relatively poor yields of recombinant protein. The need for cost-effective, simple purification schemes for biologicals intended for clinical use can not be over-emphasized.

The COS cell supernatant was purified as described *supra* and yielded approximately 1 mg of purified Ang-1-FD-Fc-FD protein that was used in the studies described *infra* to further characterize the protein.

**N-terminal sequencing of COS cell-derived Ang-1-FD-Fc-FD protein:**

Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. This was of concern because the mutant molecule, Ang1\*, has a history of containing between 10-20% N-terminally truncated species. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys<sup>1</sup>. <sup>(SEQ ID NO: 17)</sup> This sequence can be found at amino acids 16-20 of Figure 3A<sup>1</sup>, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A<sup>1</sup>. <sup>(SEQ ID NO: 6)</sup>

**Receptor binding analysis of COS cell-derived Ang-1-FD-Fc-FD:** Previous

studies have determined that the fibrinogen domain (FD) of the angiopoietin molecules is necessary for ligand/receptor interaction. Furthermore, in order for high affinity binding to the Tie-2 receptor to

weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104.

5 SDS PAGE analyses of COS cell-derived protein confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 88kD under reducing conditions (Figure 7). Light scatter analysis confirmed the molecular weight (171kD) and revealed that the Ang-2-FD-Fc-FD protein, like Ang-1-FD-Fc-  
10 FD, exists as a homogeneous species (Figure 8).

Expression Level in COS Cells: COS cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang-2-FD-Fc-FD DNA construct described  
15 *supra*. The COS cell supernatant was analyzed by Biacore to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 606, which is equivalent to 0.7mg of recombinant protein/liter of COS cell supernatant. These values represent relatively high levels of expression.

20 Purification of COS Supernatants: As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The COS cell  
25 supernatant was purified as described for Ang-1-FD-Fc-FD *supra* and yielded approximately 2 mg of purified Ang-2-FD-Fc-FD protein that was used in the studies described *infra* to further characterize this protein.

N-terminal sequencing: Purified COS cell-derived Ang-2-FD-Fc-FD protein  
30 was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Glu, wherein X is Cys. This  
(SEQ ID NO. 18)



(SEQ ID NO: 8)

sequence can be found at amino acids 16-20 of Figure 4A<sup>1</sup> and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A<sup>1</sup> (SEQ ID NO: 8)

5 **Receptor binding analysis of COS cell-derived protein:** To determine whether the COS cell-derived Ang-2-FD-Fc-FD protein could bind to the Tie-2 receptor, standard Biacore analysis was performed as described for Ang-1-FD-Fc-FD *supra*. As with Ang-1-FD-Fc-FD, a high salt wash was not  
10 Tie-2-Fc receptor, again implying that there is a strong interaction between mutant ligand and receptor.

**Example 4: Effects of COS cell-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.**

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Because Ang-1-FD-Fc-FD is a mutant molecule derived from the agonist angiopoietin-1 and Ang-2-FD-Fc-FD is a mutant molecule derived from the antagonist angiopoietin-2, we wanted to determine whether or not these two mutant molecules would retain the activity associated with the parent  
20 molecule from which it was derived.

**Assay system:** All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA 80:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill  
25 in the art.

**(A) Ang1\*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells:** EAhy926 cells were stimulated with either 0.1 µg/ml, 0.2 µg/ml, or 0.8 µg/ml Ang1\* or Ang-1-FD-Fc-FD protein.  
30 A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1\* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 9).

Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either  $dn/dc$  ( $dn$  = change of refractive index;  $dc$  = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. The results of this analysis show that the dimeric protein appears to be approximately 173.9kD and the presence of a single peak implies that the protein solution is homogenous.

**Expression level of Ang-1-FD-Fc-FD in stable CHO clones:** CHO cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-1-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of 2-3 pg/cell/day.

**Purification of Ang-1-FD-Fc-FD protein derived from stable CHO clone**

**supernatants:** Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). The CHO cell supernatant was purified as described *supra* and the purified ANG-1-FD-Fc-FD protein was used in the studies described *infra* to further characterize the protein.

**N-terminal sequencing of stable CHO clone-derived Ang-1-FD-Fc-FD**

**protein:** Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal

(SEQ ID NO. 17)  
sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be  
found at amino acids 16-20 of Figure 3A, (SEQ ID NO. 6) and immediately follows the  
protein's signal sequence corresponding to amino acids 1-15 Figure 3A. (SEQ ID NO. 6)

5 **Example 10: Characterization of stable CHO clone-derived Ang-2-FD-Fc-FD protein.**

**Molecular Weight Analysis:** As described for stable CHO clone-derived  
Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for stable CHO  
10 clone-derived Ang-2-FD-Fc-FD protein was determined using the  
MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT).  
The monomeric form of Ang-2-FD-Fc-FD has a predicted weight of 76,052,  
with three predicted N-linked glycosylation sites that could potentially  
increase the molecular weight of the monomeric protein to 83,552. Like  
15 Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular  
weight, including possible N-linked glycosylation, of 167,104. SDS PAGE  
analyses confirmed these approximate molecular weights, with a band  
running at about 200kD under non-reducing conditions and a band running  
at about 85kD under reducing conditions. Light scatter analysis confirmed  
20 the molecular weight (176.6kD) and revealed that the stable CHO clone-  
derived Ang-2-FD-Fc-FD protein, like stable CHO clone-derived Ang-1-FD-  
Fc-FD, exists as a homogeneous species.

**Expression level of Ang-2-FD-Fc-FD derived from stable CHO clones:** CHO

25 cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was  
generated by stably transfecting CHO cells with the Ang-2-FD-Fc-FD DNA  
construct described *supra*. The CHO cell supernatant was analyzed by  
standard ELISA using an anti-human IgG antibody as a capture antibody  
and an anti-human IgG antibody conjugated to alkaline phosphatase as a  
30 reporter antibody to quantitate the amount of Ang-2-FD-Fc-FD protein  
present in the supernatant. This analysis revealed expression levels of  
approximately 1-2 pg/cell/day.

Purification of stable CHO clone-derived Ang-2-FD-Fc-FD from cell

supernatants: As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The CHO cell supernatant was purified as described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra* and was used in the studies described *infra* to further characterize this protein.

N-terminal sequencing of stable CHO clone-derived Ang-2-FD-Fc-FD

protein: Purified stable CHO clone-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Asp-X-Ala-Glu-Val, wherein X is Cys. This sequence can be found at amino acids 17-21 of Figure 4A', and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A'.  
(SEQ ID NO: 19)  
(SEQ ID NO: 8)

Example 11: Effects of stable CHO clone-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.

Assay system: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA 80:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

(A) Ang1\*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-

mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were stimulated with 0.4 µg/ml Ang1\* or 0.2 µg/ml or 0.4 µg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that or stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1\* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 17).

Construction of tandem Ephrin ectodomain/Fc domain nucleic acid molecules.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into either the mammalian expression pJFE14 (Ephrin-B1-Ephrin-B1-Fc) or pMT21 (Ephrin-B2-Ephrin-B2-Fc), each with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5' end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 12: Construction of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules.

(A) Ephrin-B1-Ephrin-B1-Fc: The Ephrin-B1-Ephrin-B1-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B1 (Davis et al., *ibid.*), which corresponds to nucleotides 1-711 of Figure 14A-Figure 14B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 712-720 of Figure 14B), followed by a second copy of the ectodomain of Ephrin-B1 (corresponding to nucleotides 721-1344 of Figure 14B-Figure 14D), except that in this copy of the Ephrin-B1 ectodomain the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1345-1353 of Figure 14D),

followed by the coding sequence for the Fc portion of human IgG1  
(nucleotides 1354-2049 of Figure 14D-Figure 14E). [SEQ ID NO. 9]

**(B) Ephrin-B2-Ephrin-B2-Fc:** The Ephrin-B2-Ephrin-B2-Fc DNA molecule

consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann  
et al., 1995, Mol. Cell Biol. 15:4821-4929), which corresponds to nucleotides 1-  
675 of Figure 15A-Figure 15B, [SEQ ID NO. 11]

followed by a bridging sequence consisting of  
the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), [SEQ ID NO. 11]

followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides  
685-1270 of Figure 15B-Figure 15D), [SEQ ID NO. 11]

except that in this copy the signal  
sequence has been removed. This second copy is followed by a second Gly-  
Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), [SEQ ID NO. 11]

followed by  
the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-  
1977 of Figure 15D-Figure 15E). [SEQ ID NO. 11]

As with the angiopoietin nucleic acid molecules described *supra*, the  
bridging sequences were introduced to provide convenient restriction sites  
and to give flexibility to the junctions between the domains.

**Example 13: Expression of tandem Ephrin recombinant proteins in COS  
cells.**

COS cells were transiently transfected with either the Ephrin-B1-Ephrin-B1-  
Fc or Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules described *supra* using  
standard transfection techniques known in the art. Two days subsequent to  
transfection, the growth medium (DMEM supplemented with 100 U/ml  
penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% calf serum)  
was aspirated and replaced with serum-free medium (DMEM supplemented  
with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine). Cell  
were grown for an additional three days and then the serum-free medium  
containing the recombinant proteins was collected. Recombinant protein  
concentration was determined by performing dot blots and comparing the

phosphorylation and the relative quantities of EphB2 in the experimental conditions described *supra*.

**Results:** Both Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc were shown to be approximately as active as anti-Fc antibody-clustered Ephrin-B1-Fc in their ability to induce EphB2 receptor phosphorylation in the COS cell reporter assay. Furthermore, if either of the proteins were further clustered with the goat anti-human Fc antibody, they became even more potent in their ability to induce EphB2 receptor phosphorylation. Figure 16 shows the results of this phosphorylation assay.

**Example 15: Construction of Ephrin-B2-Ephrin-B2-Fc CHO expression vector.**

The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. 15:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B<sup>[SEQ ID NO. 11]</sup>, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B<sup>[SEQ ID NO. 11]</sup>), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D<sup>[SEQ ID NO. 11]</sup>), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D<sup>[SEQ ID NO. 11]</sup>), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E<sup>[SEQ ID NO. 11]</sup>). This molecule was subcloned into the HindIII and NotI polylinker sites in the expression vector pRG763 and was designated pRG763-m(Ephrin-B2)2-Fc. As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.